NOTES

Structure-Activity Relationships of Tetramethylpiperidine-Substituted Phenazines against *Mycobacterium leprae* In Vitro

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In a previous study of structure-activity relationships of selected phenazines against *Mycobacterium leprae* in vitro, compounds containing a 2,2,6,6-tetramethylpiperidine substitution at the imino nitrogen were most active. Therefore, the effect of substitution at the *para* positions of the phenyl and anilino groups in tetramethylpiperidine-substituted phenazines was assessed. As determined by radiorespirometry, activity in ascending order was observed in compounds substituted with hydrogens or fluorines, ethoxy groups, methyl groups, chlorines, and bromines and correlated with partition coefficients in octanol-water.

Despite its dose-limiting gastrointestinal toxicity and dose-related skin pigmentation, clofazimine (B663) remains a first-line drug in leprosy chemotherapy (1, 14). Although a wide variety of structural analogs of B663 has been synthesized (11), only a limited number of compounds have been evaluated in the 6- to 12-month mouse footpad model (13), which until recently was the only available screening system for new antileprotics. An examination of six structural analogs of clofazimine in a *Mycobacterium leprae*-macrophage in vitro system failed to identify compounds with superior activity (10).

Using a radiorespirometric metabolic assay, we have been rapidly and inexpensively evaluating a large number of antimicrobial agents for antileprosy activity in vitro (3–8). Recently we assessed 12 phenazines which (besides not being expected to accumulate in body fat) differ from clofazimine in substitution at the imino nitrogen and in some cases in chlorination of the phenazine nucleus or anilino and phenyl rings (6). In general, activity increased with the degree of chlorination. Phenazines containing a 2,2,6,6-tetramethylpiperidine substitution (TMP) at the imino nitrogen were clearly the most active compounds in the series, with the monochloro (B4019) and dichloro (B3786) analogs displaying essentially identical activity.

We describe here the in vitro assessment of substitution at the *para* positions of the anilino and phenyl groups of TMP, with a view toward identifying nonpigmenting compounds with high activity against *M. leprae*.

Susceptibility testing was performed essentially as previously described (6). Briefly, footpads of *M. leprae*-infected, athymic nude mice were surface decontaminated, and bacilli were obtained by homogenization of the footpads in Dubos-albumin medium (pH 6.5) followed by differential centrifugation. Samples were removed for contamination checks on a variety of culture media. The remaining *M. leprae* suspension was held at 4°C for 2 days, during which time the culture medium was observed to confirm the absence of contamination. The suspension was then diluted in Dubos-albumin medium to a final density of 10^7/ml and distributed in 1-ml aliquots to 6-ml glass screw-cap vials. Phenazines were

solubilized in absolute ethanol at 200 μg/ml with the aid of a sonicating bath and stored at 4°C until used. Dilutions were made in absolute ethanol, and 10 μl was added to the 1-ml cultures. Controls and heat-killed bacilli received 10 μl of ethanol. All groups consisted of quadruplicate samples, except for viable controls, for which $n = 12$. Cultures were incubated for 6 days at 33°C with loose caps in chambers which were flushed continuously with room air via an aquarium pump. [1-^{14}C]palmitic acid (1 μCi; 58 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was then added to each vial in 10 μl of ethanol, and the glass vials were placed in wide-mouth plastic scintillation vials containing a hollow cylinder of filter paper which had previously been saturated with a concentrated liquid scintillation fluor
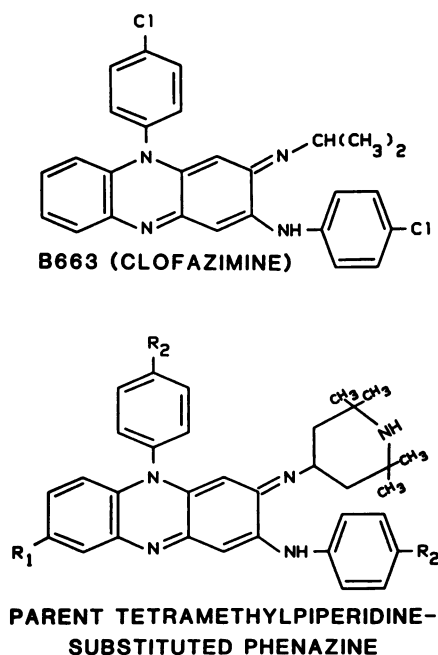

FIG. 1. Structures of B663 (clofazimine) and parent TMP.

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TABLE 1. Structure-activity relationships of TMPs against *M. leprae*

Phenazine	R_1	R_2	P^a	Mean cpm (SD) of $^{14}CO_2$ evolved after preincubation with phenazine at concn (ng/ml) of:				
				0	31	125	500	2,000
None				19,507 (1,770)				
Heat killed				32 (6)				
B663	NA^b	NA	625		17,829 (4,191)	17,735 (949)	15,445 (2,581)	1968 (493)
B3962	H	H	8		17,136 (3,160)	17,992 (481)	7,790 (507)	222 (69)
B4075	H	F	8		15,986 (2,646)	17,386 (1,802)	8,911 (1,295)	133 (13)
B4064	H	OC_2H_5	17		13,471 (2,791)	15,021 (1,338)	4,366 (1,339)	100 (6)
B4070	H	CH_3	17		17,116 (1,156)	14,619 (1,292)	1,477 (197)	23 (7)
B4019	Cl	H	27		17,304 (1,715)	4,330 (1,241)	38 (12)	24 (5)
B3786	H	Cl	26		17,217 (1,137)	5,090 (465)	45 (17)	29 (6)
B4076	H	Br	37		11,910 (566)	629 (163)	25 (6)	23 (5)

[a] P, Partition coefficient in mixture of 1-octanol and water. P = (concentration in octanol/volume of octanol)/(concentration in water/volume of water).
[b] Not applicable. See Fig. 1.

and alkalinized with 100 μl of NaOH (2). The entire assembly was then incubated at 33°C, and the evolved $^{14}CO_2$ was quantitated at 5 days after label addition by placing the assembly in a liquid scintillation counter.

For the determination of partition coefficients, phenazines were solubilized and diluted in 1-octanol (9) (high-pressure liquid chromatography grade; Aldrich Chemical Co., Inc., Milwaukee, Wis.) to 40 μg/ml. Maximum absorption of visible light was found to range from 454 to 462 nm. Two milliliters of the phenazine solutions was added to 40 ml of deionized water in a 50-ml conical styrene tube and mixed by 100 inversions at room temperature. One milliliter of the upper octanol phase was removed, and the A_{458} was read. Concentrations were determined from standard curves of phenazines in 1-octanol.

The structures of B663 and the parent TMP are shown in Fig. 1. The effects of substitution at R_2 (and in one case, at R_1) on the subsequent evolution of $^{14}CO_2$ from [1-^{14}C] palmitic acid by *M. leprae* as well as on partitioning in octanol-water are shown in Table 1.

All of the TMPs were clearly more active than B663 in reducing $^{14}CO_2$ evolution by *M. leprae*. The least active TMP contained hydrogen (B3962) or fluorine (B4075) at R_2. Increased activity was observed with an ethoxy substitution (B4064) and again in the methyl-substituted compound (B4070). Chlorine-substituted TMPs were significantly more active than the compounds mentioned above, with no difference between the monochlorinated (B4019) and dichlorinated (B3786) analogs, as noted previously (6). The bromine-substituted TMP (B4076) was the most active compound, effecting 61 and 97% reductions in $^{14}CO_2$ evolution at 31 and 125 ng/ml, respectively. In subsequent experiments (data not shown), a trichlorinated analog (B4090) with a partition coefficient of 121 demonstrated even higher activity.

Among TMPs there was a general correlation between in vitro activity and partition coefficients. B4019, a chlorinated TMP with relatively high in vitro activity and partitioning in octanol, also is the most active clofazimine analog tested to date against *M. leprae* in the mouse footpad (other TMPs have not been tested), completely inhibiting growth when administered at 0.01% (wt/wt) in the diet (Thomas M. Shinnick, personal communication). However, in a kinetic (12) mouse footpad experiment, in which a drug is administered for approximately 2 months during log-phase growth and then withdrawn, B663 remains the only phenazine to inhibit *M. leprae* at 0.001% (wt/wt). Our results suggest that this is largely due to pharmacokinetic properties, in particular lipophilicity. Unfortunately, this property almost certainly is responsible for the skin pigmentation in patients

taking B663. Indeed, mice receiving B663 at 0.001% (wt/wt) had rust-colored fat, whereas the fat of mice receiving B4019 at 0.01% was pale pink, not much darker than that of control mice (Shinnick, personal communication).

Taken together, the in vitro and in vivo studies suggest that a nonpigmenting alternative to B663 may exist in a phenazine (such as B4019, B3786, B4076, or B4090) with a combination of superior in vitro activity and reduced but adequate lipophilicity.

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